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Association of Chlorophyll with Amides on Plasticized Polyethylene Particles. Effect of Temperature on Fluorescence

By

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In a number of instances, the fluorescence of chlorophyll adsorbed with other amphiphiles to swollen polyethylene particles is reduced in intensity on cooling below ambient temperature, but is restored by further cooling to liquid nitrogen temperature. The fluorescence variation is ascribed to changes in the state of association of chlorophyll with temperature, and to changes in the efficiencies of energy trapping and of fluorescence of aggregated chlorophyll forms. The changes observed on cooling are in general reversed on rewarming. The fluorescence properties of chlorophyll adsorbed with *N*-(3-pyridyl) myristamide to particles swollen with undecane, tetradecane, and squalene are compared. In the case of squalene, where little change in the state of chlorophyll association is expected, the fluorescence spectrum changes gradually from the high-temperature to the low-temperature form as trapping becomes more efficient and internal conversion less. With tetradecane-swollen particles, fluorescence is abruptly reduced below the melting point (6 °C) of the diluent, probably because of the formation of weakly interacting pairs of chlorophyll which quench singlet excited states. With undecane-swollen articles, the reduction in fluorescence on cooling is more gradual and is probably due to trapping by an increasing concentration of associated chlorophyll forms which fluoresce less efficiently than monomeric chlorophyll. There is some dependence on the comosition of the paste in which the particles are suspended for spectral measurement: pastes which contain Carbowax, a weak ligand for chlorophyll, favor the fluorescence of less highly associated species than pastes composed entirely of carbohydrates.

Introduction

In our previous article on the subject of association of chlorophyll a with the isomeric *N*-pyridylmyristamides (NPMA's) adsorbed to swollen polyethylene (PE) particles (Part II of this series[1]), absorption and fluorescence spectra at room temperature were described in detail and analyzed into minimal numbers of components. Both absorption and fluorescence spectra contained bands assigned to associated chlorophyll species, positions and relative intensities of which were characteristic of the NPMA isomer adsorbed with it, and between which reasonable correlations could be made.

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In (II) only room temperature absorption and fluorescence were analyzed. Although the positions of the main fluorescence bands at 77 K were mentioned, detailed analysis was deferred for the following reason: it was latterly found that on particles swollen with tetradecane, fluorescence was substantially reduced at temperatures somewhat below its freezing point (6 °C), but strengthened again on further cooling. An example of this is shown in Fig. 1, for chlorophyll adsorbed with *N*-(2-pyridyl) myristamide (2-NPMA). The high-temperature fluorescence spectrum (296 K) is quite similar to that for 2-NPMA shown in Fig. 3b of (II). On cooling to 246 K, not only is the fluorescence ascribed to monomeric chlorophyll (674.5 nm, see Fig. 3b of (II)) suppressed, but the other fluorescence band components are greatly reduced. On further cooling, all components except the monomeric one increase, the greatest increase being in the 693 nm component. The changes seen on cooling are largely reversed on warming, therefore do not correspond to irreversible alteration of the mode of association of chlorophyll.

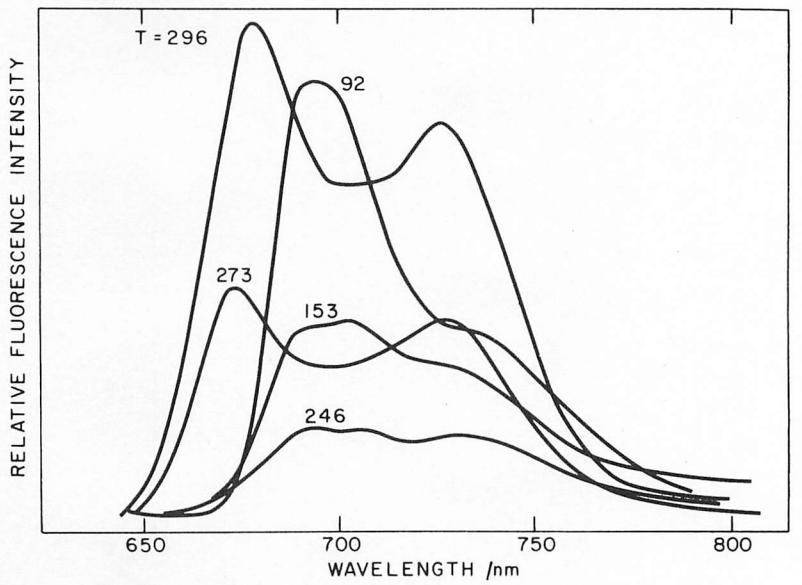

Fig. 1. Fluorescence spectra of chlorophyll (1.03 μmol g^{-1}) adsorbed with *N*-(2-pyridyl)-myristamide (75 μmol g^{-1}) to PE/tetradecane particles and suspended in a Carbowax-glycerol paste, at five temperatures excerpted from a longer series. The suppression of monomer fluorescence below the melting point (279 K) of tetradecane, and the recovery in bands other than the monomer at lower temperatures are illustrated. These spectra are not corrected for variation of instrumental sensitivity with wavelength.

If monomeric chlorophyll ligated with an amphiphile is in equilibrium with associated but still fluorescent species on the surface of the particle, the following events may be anticipated as the temperature is lowered. (1) As the temperature is reduced from ambient to the freezing point of the diluent (*e.g.*, tetradecane) in the particles, the fraction of chlorophyll in associated species should increase. (2) As the diluent freezes, sequestration of chlorophyll and amphiphile into pools of eutectic should greatly increase the fraction of chlorophyll in some associated

form. (3) On continued cooling of the solid solution, the probability of fluorescence from associated species should increase as energy trapping becomes more efficient and the processes leading to quenching by internal conversion are frozen out.

The aim of the present work was to test these expectations using particles swollen with different diluents: tetradecane, undecane, and squalene, with similar amounts of chlorophyll and amphiphile adsorbed to them. The amphiphile chosen was *N*-(3-pyridyl) myristamide (3-NPMA), which was shown to be a strong ligating ligating agent for chlorophyll.[1] It was hoped that more might be learned about the manner of association of chlorophyll and surfactant from the temperature dependence of fluorescence.

The particle diluents were chosen for their phase transition properties. The relatively high melting point (6 °C) of tetradecane contracts with the relatively low one (− 25 °C) of undecane. Squalene is a viscous triterpene which probably cools to a glass but does not freeze under our operating conditions. Our sample of squalene solidified after long storage in a freezer, and a thermometer immersed in melting squalene registered about − 15 °C.

Experimental

Materials and experimental procedures were essentially the same as described in (II),[1] with the following amplifications. The swollen polyethylene particles were prepared by dissolving polyethylene in hot undecane, tetradecane or squalene, and cooling, as described in previous publications.[1-3] Particles prepared in this way contain about 75% diluent. Chlorophyll, in amount intended to yield less than 1 μmol g^{-1} of particles, and 3-NPMA, 5 mM** in 90% methanol, were adsorbed from aqueous methanol[2] to give PE/undecane particles with $[\mathrm{Chl}]_p = 0.52$ and $[\text{3-NPMA}]_p = 8.03$ μmol g^{-1}, PE/tetradecane particles with $[\mathrm{Chl}]_p = 0.65$ μmol g^{-1} and $[\text{3-NPMA}]_p = 3.09$ μmol g^{-1}, and PE/squalene particles[3] with $[\mathrm{Chl}]_p = 0.77$ μmol g^{-1} and $[\text{3-NPMA}]_p = 4.97$ μmol g^{-1}, where the subscript "p" denotes molal concentration on particles. The 3-NPMA content evidently depends on the identity of the diluent; however, since 3-NPMA is always in excess, the state of chlorophyll association is not expected to depend on its exact concentration.

Fluorescence spectra were recorded in the apparatus previously described,[1] but with a Hamamatsu R928 photomultiplier as detector, in a Products for Research, Inc., cooled housing. The sample was held in an Air Products Company Cryo-Tip shroud which could be cooled by the Joule-Thomson effect to about the temperature of liquid nitrogen. The temperature was controlled by the nitrogen flow rate, and fluorescence spectra were recorded at stages during the cooling and rewarming cycle. The recorded spectra were corrected for instrumental sensitivity to wavelength and resolved into components as describd in (II). Total fluorescence intensity was calculated as the sum of the products of component band heights and bandwidths (B). Since it was not possible to determine quantum yields accurately, it is not possible to compare fluorescence intensities of different samples meaningfully. However, all samples examined fluoresced strongly at room temperature with yields evidently comparable to those of chlorophyll in solution.

Absorption spectra and their second derivatives were recorded as described in (II). It

** 1 M = 1 mol dm^{-3} and 1 mM = 1 × 10^{-3} mol dm^{-3} in this paper.

was not possible to record absorption spectra at temperatures below ambient with the precision of fluorescence spectra, and no attempt was made to do so. Our experience with other particle preparations is that cooling to 77 K induces little change in absorption beyond a slight sharpening of the bands.

The particles are customarily suspended in a highly scattering paste for absorption and fluorescence spectroscopy. Since the sample holder must be evacuated for low-temperature fluorescence, pastes containing much water or other volatile substances are unsuitable. Two pastes were used in this work: a mixture of 10:11:1 Carbowax (PEG-1000): glycerol: water (CGW), and a mixture of 6:10:3 Ficoll (Pharmacia, Inc.): glycerol: cellulose (Sigmacell) (FGC). Similar pastes to the first (CGW) have been used successfully before for this purpose.[1,2,4] We have, however, found evidence in several instances of a weak interaction between Carbowax and chlorophyll, which causes the latter to appear in less highly associated form in CGW than it would otherwise. Although Carbowax does not compete with NPMA's as a ligand for chlorophyll, we have guarded against misinterpretation by recording and analyzing each preparation in FGC as well, since this paste has given no indication of interaction with chlorophyll (*i.e.*, the absorption spectrum of particles suspended in FGC is the same as that of undiluted particles, as well as the latter can be determined).

In confirmation of an interaction between Carbowax and chlorophyll, particles were prepared containing chlorophyll and Brij 96 (poly (oxyethylene) 10 oleyl ether), which has the same ether function as Carbowax. The absorption spectrum of a suspension in 8.3:6.6 mannitol: glycerol showed one band peak in the red at 668.5 nm, whose position, width (26 nm) and second derivative indicate moderate chlorophyll association. In a 5.8:2.5:1.8 Ficoll: water: cellulose paste the spectrum was similar but a band at 741 nm was present, indicating that water had replaced ether as a ligand for some of the chlorophyll molecules. The fluorescence

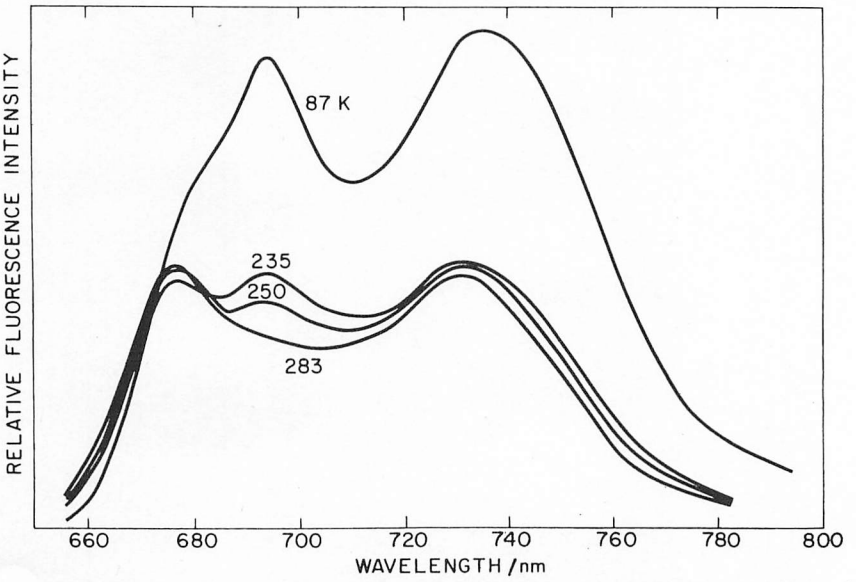

Fig. 2. Fluorescence spectra of chlorophyll adsorbed with 3-NPMA to PE/squalene particles, and suspended in FGC, at four temperatures. These and subsequent spectra are corrected for variation of instrumental sensitivity with wavelength.

in mannitol: glycerol had one band at 668 nm (probably corresponding to a 662 nm component in the absorption spectrum) and a strong band at 725 nm.

Results

It was possible to resolve all fluorescence spectra into rather similar sets of components, most of which shift toward the red as the temperature decreases. All sets of spectra feature three distince bands, of monomer fluorescence in the 671–676 nm region, and of associated chlorophyll species in the 685–692 and 727–730 nm regions, where the exact location of the band varies with particle diluent, suspending medium, and temperature. There is some uncertainty as to the proper resolution in the 695–720 nm region, where at least one and sometimes three components appear required. Quite possibly there are a number of bands in this region, and what we have obtained is just the simplest resolution that can represent the spectrum in this region with reasonable accuracy. It is also a region in which monomeric chlorophyll has a weak vibrational satellite band.[1] The red band around 760 nm in most of

Table 1. Distribution of Fluorescence Intensity among Components, for Chl a with 3-NPMA on PE/Squalene Particles Suspended in CGW and FGC

CGW						
	λ/nm:[a]	676	691	705	730	754
	B/nm:	20	16	19	35	20
T/K		Percent of total fluorescence intensity				
295		32.1	13.8	10.6	40.6	2.9
287		30.4	11.3	10.9	41.4[b]	6.0
274		28.1	14.6	9.9	42.5	4.9
249		29.6	10.1	11.3	43.6	5.4
233		26.9	13.5	10.4	43.8	5.4
221		25.3	14.0	10.3	43.8	6.6
123		10.7	25.0	8.0	52.8	3.6
	λ/nm:	676	692	710	736	760
	B/nm:	17	20	19	37	18
FGC						
	λ/nm:	675.5	692	705	730	754
	B/nm:	22	16	19	35	23
T/K						
283		29.0	8.7	10.0	45.4	6.9
235		22.2	12.9	10.5	47.5	7.0
87		15.5	13.4	7.5	57.1	6.5
	λ/nm:	678.7	693.5	706	735	766
	B/nm:	20	16	19	44	35

a) The position (λ) and bandwidth (B) of each component are listed at the highest and lowest temperature reported. Positions and bandwidths at intermediate temperatures are given fairly well by interpolation.

b) Includes a minor component at 747.5 nm, B = 14 nm, 2.3%.

the resolutions simply collects vibrational satellites of bands at higher energies and is unlikely to contain fluorescence of a distinct species.

Fluorescence spectra of chlorophyll on PE/squalene particles were very similar whether suspended in FGC or CGW, and total fluorescence intensity did not decrease significantly on cooling. In either paste, as the sample is cooled the strengths of the 692 and 730 nm

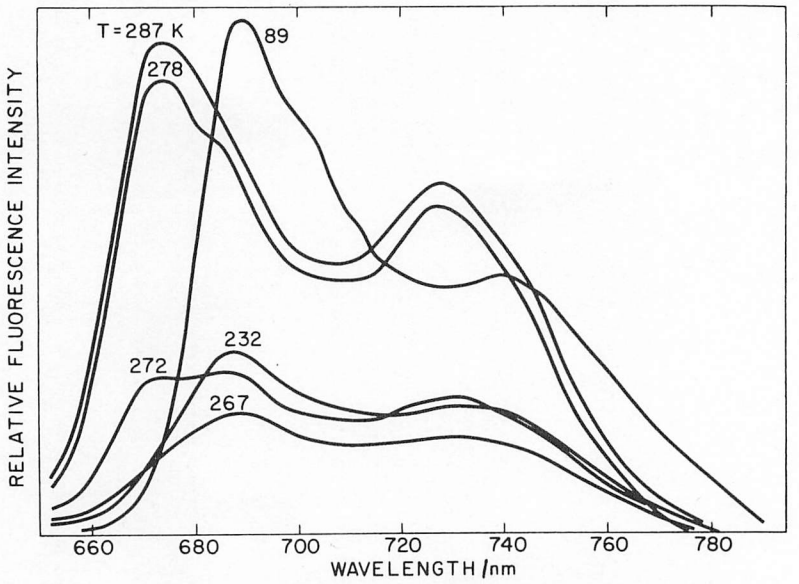

Fig. 3. Fluorescence spectra of chlorophyll adsorbed with 3-NPMA to PE/tetradecane particles, and suspended in CGW, at several temperatures.

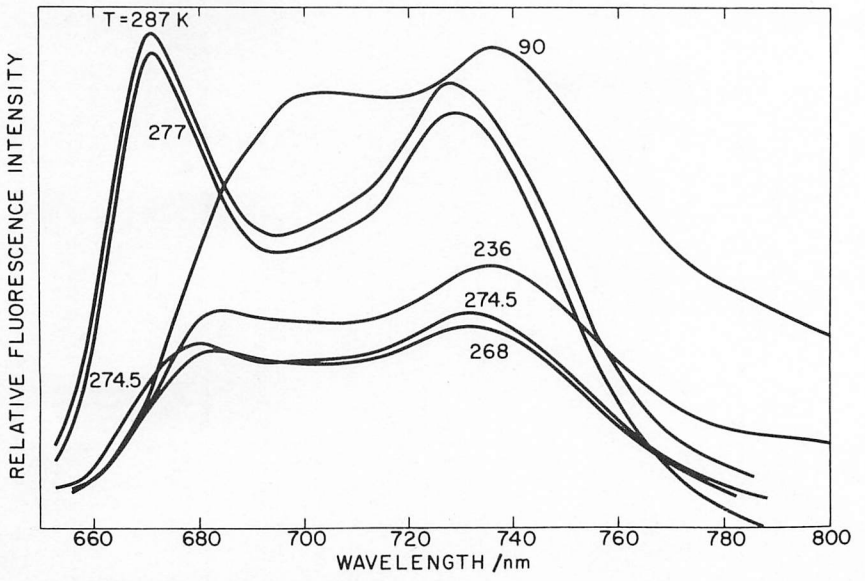

Fig. 4. Fluorescence spectra of the same particle preparation as in Fig. 3, but suspended in FGC.

components increase steadily, as shown in Fig. 2 for the FGC suspension. The analysis of the distribution into components (Table 1) shows little change from 295 to 221 K, beyond a gradual decrease in the monomer component (676 nm). All components are somewhat red-shifted with respect to those in PE/tetradecane and PE/undecane particles, which is probably a medium effect of the more polarizable squalene.

Although there is little change in the fluorescence of chlorophyll on PE/tetradecane particles above the melting point (279 K), there is marked quenching below this point, not only of the monomer component (671 nm) but of the other component bands as well. This is true in both CGW and FGC; however on further cooling, it is the 688 nm component that gains most in intensity in CGW, whereas in FGC, it is components in the 695–705 nm region. The spectral changes in CGW and FGC suspensions are shown in Figs. 3 and 4; analyses into

Table 2. Distribution of Fluorescence Intensity among Components, for Chl a with 3-NPMA on PE/Tetradecane Particles Suspended in CGW

	λ/nm:	671.8	685.7	698	709		727	751
	B/nm:	19	15.8	19	14		38	31
T/K		Percent of total fluorescence intensity						
287		28.0	12.4	10.8	0.9		41.5	6.4
278		29.1	12.1	10.9	1.2		43.3	3.5
	λ/nm:[a]	671	687.8	703	713	725.5	733	769
	B/nm:	20	18	18	19	16	45	22
272		23.1	18.0	8.6	1.8	1.2	46.5	0.7
267		13.3	25.5	7.1	7.3	1.9	44.8	0
231.5		9.1	28.4	7.6	7.7	1.0	45.8	0.5
89		0	36.0	9.5	9.7	0.9	39.9	4.1
	λ/nm:	674	688.9	703	713.5	726	739.5	773.5
	B/nm:	19	19.5	13	16	10	42	28

a) The set of components employed above the melting point of tetradecane could not be adapted to fluorescence below the melting point. The 725.5 nm component, which decreases witn temperature, may include a vibrational satellite of the monomer component (671 nm).

Table 3. Distribution of Fluorescence Intensity among Components, for Chl a with 3-NPMA on PE/Tetradecane Particles Suspended in FGC

λ/nm:	670.3	684.8	695.7	705	715.5	728	764
B/nm:	19	15.5	15	14	15.5	41	44
T/K	Percent of total fluorescence intensity						
287	24.7	8.4	5.7	3.3	–	49.0	8.9
277	25.1	9.2	5.4	3.2	–	53.6	3.5
274.5	14.1	10.6	7.3	4.1	0.9	52.5	10.6
268	11.9	11.7	8.0	4.5	1.8	53.3	8.8
236	8.5	10.2	7.5	4.8	2.3	50.6	16.6
90	9.0	7.4	8.3	5.6	3.2	47.9	21.7
λ/nm:	675	686	696.5	706	715.5	735	779
B/nm:	17	15	15	15	15.5	46	46

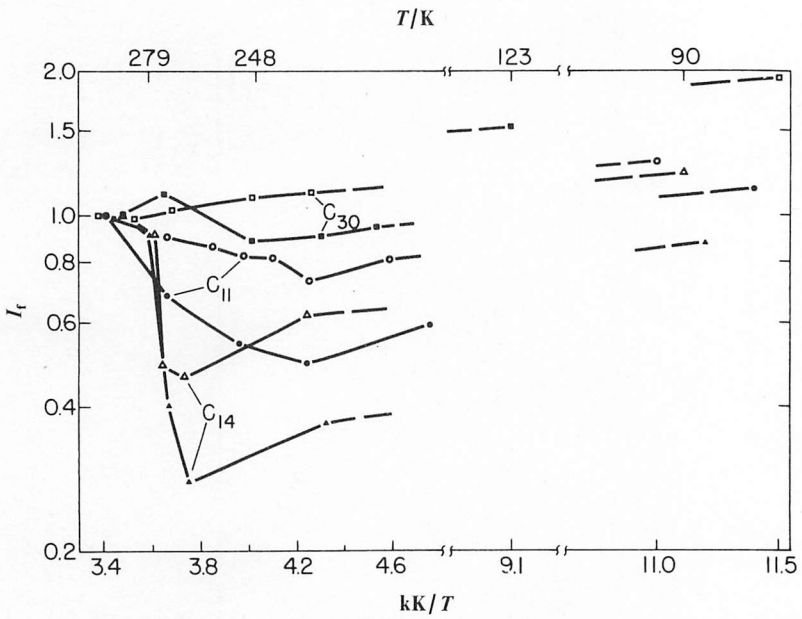

Fig. 5. Plots of total integrated fluorescence intensity (I_f, on logarithmic scale), normalized to 1 at the highest temperature recorded, against reciprocal temperature for chlorophyll with 3-NPMA on PE/squalene (C_{30}), PE/undecane (C_{11}), and PE/tetradecane (C_{14}), suspended in FGC (open symbols) and CGW (solid symbols). The melting points of tetradecane (279 K) and undecane (248 K) are indicated. The slopes of the dashed lines connecting the last two points of each set are drawn to scale.

components are presented in Tables 2 and 3. Below 275 K a new band component appears, at 713 nm in CGW and 715.5 nm in FGC, which strengthens rapidly on further cooling; perhaps it represents a chlorophyll species formed on freezing which is almost nonfluorescent at high temperature.

On PE/undecane, total chlorophyll fluorescence intennsity diminishes steadily as the temperature is lowered to about 235 K, 13 K below the melting point of undecane, then increases on further cooling. This behavior is compared with that for PE/squalene and PE/tetradecane particles in Fig. 5. There is no certain indication of an abrupt drop in total fluorescence intensity below the melting point, as there was with tetradecane. The analysis into components shows that down to about 235 K, fluorescence intensity is transferred from the 673 nm monomer component to the 688 nm associated species, in both CGW (Table 4) and FGC (Table 5). On further cooling, the fraction of fluorescence which is monomeric continues to decrease, but otherwise the apportionment changes little. The contribution assigned to 705 nm fluorescence becomes greater in FGC suspension than in CGW, as was the case for PE/tetradecane particles.

The fraction of fluorescence belonging to monomeric chlorophyll is plotted against $1/T$ in Fig. 6 for the six samples examined. The decrease in this fraction resembles the decrease in total fluorescence (Fig. 5) of PE/undecane and PE/tetradecane at the higher temperatures, but unlike the total fluorescence the monomer component fraction does not intensify at lower temperatures.

Table 4. Distribution of Fluorescence Intensity among Components, for Chl a with 3-NPMA on PE/Undecane Particles Suspended in CGW

λ/nm:	673	688	702	718	730
B/nm:	22	18	16	12	47
T/K	Percent of total fluorescence intensity				
293	30.9	15.9	6.6	0.7	45.8
273	18.2	22.2	8.5	2.3	48.8
253	13.8	27.8	8.0	—	50.4
236	12.1	28.9	10.3	—	48.8
210	11.3	28.2	10.9	—	49.6
88	3.9	30.0	8.7	3.6	53.8[a]
λ/nm:	671	689	705	717	736
B/nm:	20	21	15	15	49

a) In this spectrum, there was a substantial contribution from a component near 770 nm, which was not included in the calculation.

Table 5. Distribution of Fluorescence Intensity among Components, for Chl a with 3-NPMA on PE/Undecane Particles Suspended in FGC

λ/nm:	672.4	688	702	715.5	729	760
B/nm:	22	19	18	14	40	32
T/K	Percent of total fluorescence intensity					
293	32.2	15.7	7.6	0.8	38.2[a]	5.2
273	22.3	17.4	8.8	1.2	43.6	6.7
260	22.6	17.6	8.3	1.8	43.0	6.6
251	18.0	22.5	8.1	1.8	42.6	7.1
244	18.1	21.5	10.0	1.2	43.0	6.2
235	15.0	23.7	10.9	0.9	42.6	6.9
218	15.2	21.2	11.8	0.2	41.2[b]	10.0
91	9.2	20.4	13.6	0.5	44.7	11.5
λ/nm:	674	689	705	716	735	767
B/nm:	24	20	24	18	43	43

a) Includes a minor component at 745 nm, B = 10 nm, 0.3%.
b) Includes a minor component at 725 nm, 0.4%.

As to the actual intensities of fluorescence components, these are plotted against $1/T$ in Fig. 7 for the typical case of chlorophyll and 3-NPMA on PE/undecane in FGC. After some uncertainty at the higher temperatures, the intensities of all components except the monomeric (672–674 nm) increase with $1/T$ with similar, though not identical, slopes. In this example the fractional increase in the 702–705 nm component is largest; in CGW suspensions it is the component near 690 nm that increases most. In contrast, with one exception (PE/tetradecane in FGC) the monomer component decreases on lowering temperature, in absolute terms as well as relative as shown in Fig. 6. If the increases could be ascribed to a single process of thermally activated internal conversion, they would correspond to no more than 1–2 kJ in

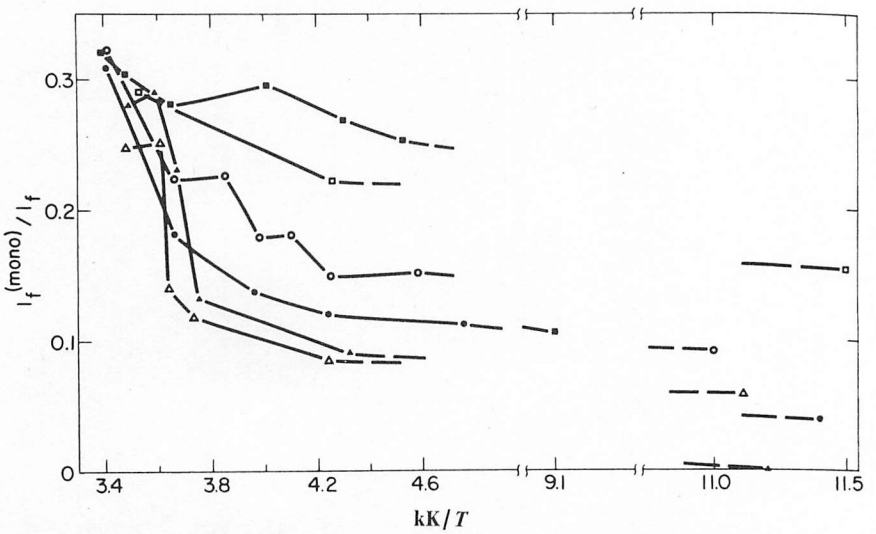

Fig. 6. Plots of the ratio of monomer component to total fluorescence intensity (from Tables 1–5) against reciprocal temperature for the six samples of Fig. 5. The same notation is used as in Fig. 5. Unlike the total fluorescence, the monomer component fluorescence does not recover at low temperature.

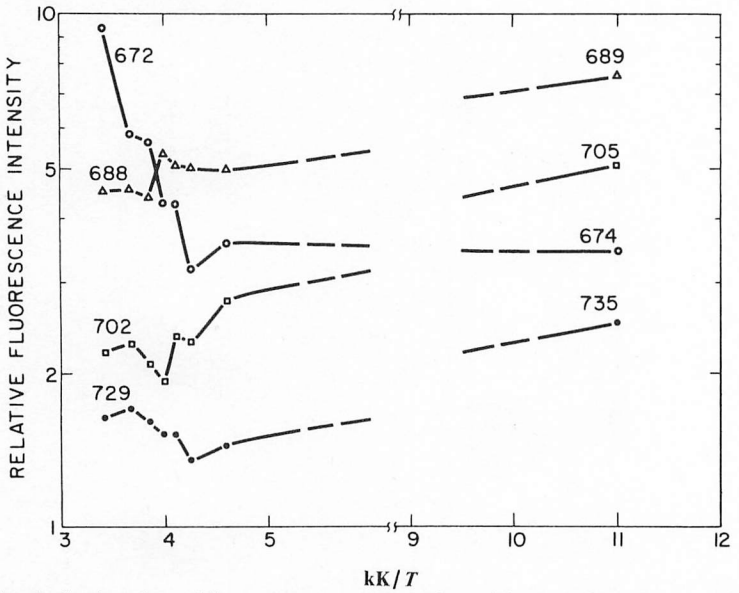

Fig. 7. Arrhenius plots of fluorescence component intensities for chlorophyll and 3-NPMA on PE/undecane, suspended in FGC. The monomeric (672–4 nm) component, and the associated species components (688–9, 702–5, and 729–35 nm) are designated by their positions at high and low temperatures (see Table 5). Points for the 729–35 component are plotted at one-tenth their actual value, compared to the others. Slopes of lines connecting points at the two lowest temperatures are drawn to scale.

most cases, similar to that estimated for chlorophyll adsorbed with *N*-methylmyristamide on PE/tetradecane, which shows a remarkable increase in fluorescence intensity on cooling.[4]

N, N-Dimethylmyristamide (DMMA). The phenomenon of quenching and recovery of fluorescence on cooling is not confined to preparations with NPMA's. We also examined the temperature variation of fluorescence of chlorophyll ($[Ch1]_p = 1.08\ \mu mol\ g^{-1}$) adsorbed with the above amphiphile ($[DMMA]_p = 29\ \mu mol\ g^{-1}$) on PE/tetradecane particles suspended in Carbowax-glycerol. Absorption and fluorescence spectra of chlorophyll and DMMA on PE/undecane were described at some length in Part I of this series.[2] The present analysis procedure yielded seven components: 671.5, 678, 694, 704, 717, 726, and 748 nm at room temperature, the first four of which and the 726 nm band had been identified under various conditions in (I). On cooling below the melting point of tetradecane, the total fluorescence falls by about 40%, distributed among all bands, but most strongly affecting the monomer component (673 nm). On further cooling all components except the monomer increase; and at 77 K the components, ranked in order of magnitude, appear at 686, 736, 698, 707, 676, 718.5, and 766 nm. The results are therefore similar to those with 3-NPMA.

Discussion

Analysis of the fluorescence spectra of these samples led, as in (II), to the identification of component bands near 673, 688, and 730 nm at room temperature in every case, and with near unanimity to at least one more component near 705 nm. The 673 nm band is assigned to monomeric chlorophyll, the 688 nm band to an associated (probably dimeric) species, and the 705 nm band to another, probably more highly aggregated species. In (II) as in (I) the broad fluorescence band near 730 nm was ascribed to excimer emission, perhaps from a pair of molecules absorbing near 653 and 674 nm, or alternatively to a postulated broad but very weak absorption band presumed to lie around 700 nm, but not observed. The discussion of energy transfer to follow will support the former assignment.

The results of the fluorescence analysis largely fulfill the expectations set out at the beginning. Our interpretation of them is as follows.

PE/Squalene. Lateral diffusion is slow in the viscous diluent and there is little opportunity for chlorophyll to form new associations as the temperature is lowered. The decrease in monomer (676 nm) fluorescence probably reflects more efficient trapping by the 692 nm species. The increase in all components except monomer at low temperature must reflect suppression of thermally activated internal conversion.

PE/Tetradecane. As the diluent freezes, chlorophyll and 3-NPMA would be crowded into small puddles of eutectic. Under these forced conditions, associations may form that are not of the sort that prevails above the melting point. The sudden decrease of all fluorescence components, not just the monomeric, indicates the formation of weakly interacting pairs of chlorophyll similar to those postulated in concentrated solutions,[5] lecithin films,[6] polymer adsorbates,[7] and liposomes.[8] The absorption spectrum of the weakly interacting chlorophylls differs little from that of isolated pigment,[7] but the excited singlet state is quickly quenched through nonradiative excimer formation or reversible electron transfer.[8-10] Apparently, the quenching process in the "interacting pairs" is itself quenched at lower temperatures, because

the recovery of fluorescence is steeper than with PE/squalene and PE/undecane particles (Fig. 5).

PE/Undecane. On cooling particles with this most fluid diluent, more chlorophyll associates into species fluorescing at 688 and 702 nm, so that there is less to form "interacting pairs" when the freezing point is reached. Also, the trends for PE/tetradecane in Fig. 5 suggest that quenching is becoming less effective anyway at this temperature.

CGW. The influence of this suspending medium is apparent in PE/tetradecane and PE/undecane preparations, where the 688 nm component is strengthened at low temperatures at the expense of the presumably more highly associated species fluorescing in the 695–720 nm region. There is no obvious effect on the 730 nm component. The use of CGW apparently affects the numbers, but not the natures, of associated chlorophyll species.

Comparison with Other Model Systems.

Many accounts have been published on chlorophyll fluorescence at room temperature and at liquid nitrogen temperature, but data for the temperature range between are vanishingly few. The only system examined with similar intent to ours is that of chlorophyll in vesicles. Chlorophyll in dipalmitoylphosphatidylcholine vesicles exhibits an abrupt drop in fluorescence when the temperature is lowered through a phase transition at 41 °C.[11,12] The fluorescence change is sensitive to chlorophyll concentration in vesicles,[12,13] as is the state of organization of chlorophyll.[14] Although the chlorophyll absorption spectrum shows components in addition to that of the monomer,[12] the fluorescence spectrum apparently does not.[11,12,15] Fluorescence intensity becomes strongly quenched at higher chlorophyll concentration, probably by the aggregated species present.[12,13]

Litvin and associates have looked for and detected many component bands in the absorption and fluorescence spectra of chlorophyll in concentrated solutions, and in films case from them.[16] Most work seems to have been done only at 20 °C and − 196 °C; however, Litvin and Gulyaev reported that the short-wave components (682, 696 nm) of the fluorescence of chlorophyll in monolayers intensified gradually as temperature decreased, while the long-wave components (724, 738, 752 nm) intensified rapidly below about − 140 °C.[17]

Our system differs from the vesicle system in a number of respects, *e.g.*, it is the diluent, not the surfactant, that freezes. Our flurescence is strong and complex at much higher ratios of chlorophyll to surfactant than in the vesicle system, though this is not necessarily an instructive comparison, since vesicle systems generally lack diluents. Fluorescence has not been investigated in vesicle systems below the freezing point of water so there is no information as to recovery at lower temperatures. Our results show many interesting parallels to the results reported by Litvin and associates, though it is difficult to identify a basis for a detailed comparison.

The increase in fluorescence intensity of chloroplasts and algae on decreasing temperature is well known, however. Photosystem fluorescence bands at 680, 685, 695, and *ca.* 735 nm intensify greatly at temperatures down to and below 77 K.[18]

Models of Energy Transfer. One of the expectations of the present undertaking is some insight into the way chlorophyll is arranged in the particulate system. The rate and extent of energy transfer, if known, is a valuable probe of the system structure. From the constitution

of the system energy transfer is expected to be rapid and extensive, but that has not hitherto been clearly established. The results of the present work discount two extreme models of chlorophyll organization, and support a third.

(1). *Boltzmann Distribution Model*: In this concept, each possible excited state of the ensemble is always in equilibrium with all the others, so that the population and therefore the fluorescence yield of each is determined by a Boltzmann partition function. This concept is realized if energy transfer is extremely fast compared to excited state decay. It predicts that when the temperature is low enough, all energy will drain into the lowest excited state available, presumably that giving rise to 730 nm fluorescence, regardless of the relative numbers of fluorescing species. However, as often as not, it is one of the higher energy fluorescing species that increases the most as temperature is lowered. and the increase of the 730 nm component is never disproportionately large. Therefore the energy trapping species must not be in a Boltzmann equilibrium with each other, at least at low temperature.

(2). *Isolated Species Model*: In this concept, there is no energy transfer at all, and the relative fluorescence intensities observed are products of the species populations and their respective quantum yields, at all temperatures. This concept would be realized if chlorphyll is distributed throughout the volume of the particle, rather than just on its surface. Above the melting point of the diluent, changes in the fluorescence spectrum would reflect mainly changes in the state of association of chlorophyll; below the melting point, they would reflect changes in quantum yields. This model accounts for much that is observed, but it fails to account for the fact that in most cases monomer fluorescence continues to decrease, in absolute as well as relative terms, as the temperature falls below the melting point. It is also hard to reconcile with the observed quenching of all fluorescence bands in PE/tetradecane systems just below the melting point, and their recovery at lower temperatures, because it makes no provision for interception and quenching of energy by "interacting pairs." Finally, it is completely incompatible with data on chlorophyll b - chlorophyll a systems, which will be presented elsewhere. This concept must therefore be rejected also.

(3). *Semi-isolated Domain Model*: The concept which best serves to account for all observations is that of a relatively small number of energy sinks scattered about the surface, which trap energy with high probability from a larger number of monomeric chlorophylls in domains around them. There may also be domains of monomeric chlorophyll without sinks, which can emit only the monomer fluorescence component. Back transfer is possible for higher-energy sinks, such as 688 nm, at high temperature, but becomes unlikely for deeper sinks. The domains may corespond to actual heterogeneities in the polyethylene-diluent-particle surface structure, or may be merely statistical.

This concept, like the previous one, implies that at low temperature the fluorescence spectrum reflects the relative number of sinks or domains present and their intrinsic quantum yields, irrespective of their relative energies. Above the melting point of the diluent, the shift of fluorescence to longer wavelengths reflects increased efficiency of trapping, and an increasing number of fluorescent sinks. Freezing produces "interacting pairs" distributed randomly among the domains, which intercept and quench singlet state energy before its capture by the sinks. Below the freezing point, increased fluorescence of the traps implies a greater radiative

quantum efficiency. According to this model most of the chlorophyll is likely to remain monomeric even at low temperature.

We have attempted to formulate a mathematical description of this model, and this is sketched in the Appendix. After some attempts to derive numerical information from it, it was recognized that there are simply too many unknown quantities which must be entered into the formulation to permit extraction of reliable estimates from the data available. In a simpler system the quations may be soluble, but not in a system where there may be five fluorescent species. The formulation does seem to account for observations qualitatively in a straightforward way, and we believe it is essentially correct.

The model does support an excimer assignment to the 730 nm fluorescence component, for the following reason. The distribution of fluorescence at low temperature implies that almost half of the sinks present in any of the preparations belong to this species. If the fluorescence were being emitted by a species postulated to have a broad absorption band around 700 nm, that species could not have escaped discovery by the spectral analysis results reported in (II). Indeed, the assignment to a 700 nm absorbing species is conceivable only under the premises of model (1) above,[2] which are contraindicated by the present results.

Appendix

A simplified attempt to formulate mathematically the concepts of the semi-isolated domain model begins with division of chlorophylls into domains about each of a number of energy-trapping and fluorescent sinks. Let P_0, P_1, P_2, and P_3 be the probabilities that absorbed energy will encounter no sinks, and the 688, 705, and 730 nm fluorescing sinks. If Φ_0, *etc.*, are the quantum yields of the respective fluorescences then at absolute zero the observed component quantum yields would be $P_0\Phi_0$, *etc.* Our task is to formulate the problem so as to bring out the temperature dependence of component quantum yields. It is assumed that the relative numbers of sinks do not change with temperature, so that the expressions are really only valid below the melting or softening point of the particles.

The singlet state energies of monomeric chlorophyll and of the sinks may be calculated from the fluorescence band position and bandwidth by the Stepanov relation.[19] Energy entering the 705 and 730 nm sinks is effectively trapped, and is unlikely to return to the monomer chlorophyll. Energy may return from the 688 nm sink to the monomer (672 nm) with a probability dependent on temperature. Its fate is formulated thus. The 688 nm sink is considered to be a 688 nm-fluorescing trap surrounded by a small number n of 672 nm-fluorescing monomers, such that the probabilities $P(672)$ and $P(688)$ that the energy resides on each are connected by a Boltzmann equilibrium (A1).

$$\frac{P(672)}{P(688)} = n \exp\left[-(\varepsilon_0 - \varepsilon_1)/kT\right] = n \exp\left(-515/T\right). \tag{A1}$$

A photon in the 688 nm sink has a probability P_{11} of decaying on the 688 nm species, a probability P_{10} of decaying on the monomer species, and a probability P_r of escaping the sink altogether, to be redistributed with the probabilities P_0, P_1, P_2, and P_3. Consequently the probabilities that the singlet state energy will ultimately decay on monomer and in the traps are $(P_0 + P_1P_{10})/(1 - P_1P_r)$, $P_1P_{11}/(1 - P_1P_r)$, $P_2/(1 - P_1P_r)$, and $P_3/(1 - P_1P_r)$.

The quantum yield of monomer florescence is expressed by

$$\Phi_0 = k_f/(k_f + k_d) \tag{A2}$$

where k_f is the rate of radiative decay, and k_d the sum of rates of all dark decay processes. Since monomer fluorescence varies little with temperature, k_f and k_d are taken as constant. The increase in trap fluorescences at lower temperature indicates that there are thermally activated internal conversion processes, and the quantum yields of trap fluorescence therefore should be expressed as

$$\Phi_1 = k_f^1/[k_f^1 + k_{d0}^1 + k_{d1}^1 \exp(-\Delta h_1/kT)] \tag{A3}$$

where k_{d0}^1 is the rate of nonradiative decay at absolute zero and $k_{d0}^1 + k_{d1}^1$ the rate at high temperature, and similarly for Φ_2 and Φ_3.

Since $P(688) = P_{11}$ and $P(672) = P_{10} + P_r$, one obtaines eventually

$$\begin{aligned} P_r &= k_r n \exp(-515/T)/[k_f^1 + k_{d0}^1 + k_{d1}^1 \exp(-\Delta h_1/kT) + n(k_f + k_d + k_r)\exp(-515/T)] \\ &\equiv [k_r n \exp(-515/T)]/D \end{aligned} \tag{A4}$$

where k_r is the rate of escape of energy from the sink.

$$P_{10} = [(k_f + k_d) n \exp(-515/T)]/D. \tag{A5}$$

$$P_{11} = [k_f^1 + k_{d0}^1 + k_{d1}^1 \exp(-\Delta h_1/kT)]/D. \tag{A6}$$

When the expressions for the probabilities of decay, amplified by Eqs. A4-A6, are multiplied by their respective quantum yields, Eqs. A2 and A3, the following equations result.

$$\Phi_0 = \frac{k_f}{k_f + k_d}\left[P_0 + \frac{P_1(k_f + k_d + P_0 k_r)}{(k_f + k_d + (1 - P_1)k_r) + \dfrac{k_f^1 + k_{d0}^1 + k_{d1}^1 \exp(-\Delta h_1/kT)}{n \exp(-515/T)}}\right], \tag{A7}$$

$$\Phi_1 = \frac{P_1 k_f^1}{k_f^1 + k_{d0}^1 + k_{d1}^1 \exp(-\Delta h_1/kT) + n[k_f + k_d + (1 - P_1)k_r]\exp(-515/T)}, \tag{A8}$$

$$\Phi_2 = \frac{P_2 k_f^{(2)}}{k_f^{(2)} + k_{d0}^{(2)} + k_{d1}^{(2)} \exp(-\Delta h_2/kT)}\left[\frac{1 + \dfrac{n(k_f + k_d + k_r)\exp(-515/T)}{k_f^1 + k_{d0}^1 + k_{d1}^1 \exp(-\Delta h_1/kT)}}{1 + \dfrac{n(k_f + k_d + (1 - P_1)k_r)\exp(-515/T)}{k_f^1 + k_{d0}^1 + k_{d1}^1 \exp(-\Delta h_1/kT)}}\right], \tag{A9}$$

and analogously for Φ_3.

The formulas are really appropriate only for cold PE/squalene particles. To include the quenching by "interacting pairs" would require additional expressions similar to those for P_1 which would even further complicate the expressions for quantum yield. The main point is that temperature dependence, in the form of heats of activation Δh_1 and Δh_2, does not appear in the expression in an easily factorizable manner. The equations may be useful in some analogous but simpler system, but they contain too many unknown quantities to be useful in the present case.

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